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The goal of this project is to identify members of the MDC protein family that are expressed in breast cancer tissue, to develop novel antibodies to them, and to determine their distribution in tumor tissue as well as in cultured breast cancer cell lines. As a model system for breast cancer progression, we are using transgenic mice that express the polyomavirus middle T oncogene in the mammary gland epithelium. Using the polymerase chain reaction (PCR), we have shown that MDC9 and MDC15 are expressed in mammary tumor tissue derived from these mice. We have raised polyclonal antibodies to synthetic peptides and recombinant proteins, but these antibodies did not recognize the cognate antigens in mammary tumor tissue, presumably due to lack of cross-reaction with the native proteins. We have also raised panels of rabbit monoclonal antibodies to proteins expressed on the surface of mammary tumor cells, and have identified several of the corresponding antigens. We are continuing to screen these antibodies for reactivity with MDCs. In addition, we are using DNA immunization as a direct approach to raising anti-MDC monoclonal antibodies.

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FOREWORD

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Title: Metalloprotease/disintegrin proteins and mammary carcinoma progression

INTRODUCTION

A novel family of cell surface proteins with a conserved domain structure, consisting of metalloprotease, disintegrin, and cysteine-rich domains, has recently been recognized. These proteins are termed MDC proteins or ADAMs. Their extracellular portions contain a domain related to matrix-degrading metalloproteases (MMPs), and another domain similar to integrin-binding snake venom peptides (disintegrins) (Schlondorff and Blobel, 1999). These domains are linked to transmembrane domains and short (15-100 amino acids) cytoplasmic domains. Some of the cytoplasmic domains contain consensus SH3-binding sequences, suggesting that MDCs can interact with cytoplasmic signaling pathways (Howard et al., 1999). Sequencing of cDNAs cloned from various mammalian tissues suggests that the MDC family is very large and consists of more than 30 members that are expressed in a variety of mammalian tissues. One member of this family, termed fertilin, is expressed on the outer sperm membrane and is known to be involved in sperm-egg fusion. Fertilin has been shown to interact with the $\alpha 6\beta 1$ integrin on the egg surface. One of the known MDCs, termed metargidin, contains an RGD sequence in its disintegrin domain and was reported to interact with the integrin $\alpha v\beta 3$ (Zhang et al., 1998). Since the disintegrin domains of all MDCs have a highly conserved domain structure, it appears likely that all of them can interact with integrins. Several of these proteins have now been shown to have specific metalloprotease activity, and to function in crucial regulatory events, such as the activation of TNFα (TACE) or the Notch protein (Kuzbanian).

Based on the intriguing structural properties of the MDCs, we hypothesize that they may be important regulators of cell adhesion, migration, and invasion, and that specific members of this family may contribute to the invasive growth of breast cancer cells. The distribution and possible function of these proteins in normal and malignant breast tissue has not been studied. We propose to use PCR cloning in order to identify known and novel MDCs that are expressed in human and mouse mammary tumors. We will then use in situ hybridization and immunohistochemistry to determine the distribution and expression levels of individual MDCs in normal mammary gland tissue and at different stages of mammary carcinoma progression. As a mouse model of mammary tumorigenesis, we will use transgenic mice overexpressing either the polyoma middle-T or the c-neu oncogene under the control of the MMTV promoter. In addition, we will raise polyclonal and monoclonal rabbit antibodies to synthetic peptides or recombinant protein fragments based on MDC sequences. We will use these antibodies to determine the distribution of MDC proteins in human and mouse mammary tumors. We will also use antibodies to determine the subcellular distribution of MDCs in cultured cells. This will be a first step toward identifying possible interactions with integrins localized in focal contacts or at cell-cell borders. These studies will provide the foundation for further functional studies on the possible role of MDCs in breast cancer progression in vivo.

BODY:

Mouse models of breast cancer progression: We have extended our work on characterizing two different transgenic mouse models of breast cancer. The first model expresses the polyoma middle T antigen under the control of the MMTV promoter (Guy et al., 1992). We have established colonies of these mice and have found that 100% of females develop invasive adenocarcinomas of the mammary glands by 60 days of age. Mice are sacrificed at 100 days of age, at which time 95% of them have developed extensive metastatic lesions in the lungs. Thus, this mouse strain provides a practical experimental model because of the rapid and consistent progression of malignancy. However, they may not provide a realistic model of human breast cancer, which develops stochastically and involves multiple steps of progression through genetic instability and random mutations. As an alternative model, we have also established colonies of a mouse strain expressing the c-neu oncogene under the control of the MMTV promoter. We found that female mice of this strain develop tumors much more slowly (5-8 months of age) and stochastically. Most of the tumors are invasive and metastasize to the lung by 12 months of age. We have collected tissues from normal mammary glands, early and late stage primary tumors, and lung metastases obtained from both of these mouse strains. In addition, we have established a series of cell lines from both tumor models. We are also routinely producing primary cell cultures from these tumors.

Identification of MDC proteins by PCR: We have synthesized a series of oligonucleotide primers designed to hybridize with conserved sequences in the metalloprotease domains and in the disintegrin domains of various MDC proteins. These primers were either degenerate, i.e., expected to hybridize with most or all members of the MDC family, or specific for individual members of the family (MDC9, MDC10, MDC11, MDC12, MDC15). Primers were designed to recognize the nucleotide sequences encoding the following conserved amino acid sequences in the metalloprotease domain and the disintegrin domain, respectively:

Primer 1: MXHEXGHN (metalloprotease domain) (forward orientation)
Primer 2: EECDCG (forward orientation)
Primer 3: ECDLXEXC (reverse orientation)

We have used these primers for PCR amplification of total cDNA obtained by reverse transcription of mRNA purified from mouse mammary tumor tissues. These tissues were derived from primary tumors and metastatic lesions arising in MMTV-middle-T transgenic mice, or MMTV-c-neu transgenic mice. When degenerate primers were used, bands of the expected size (200 bp for primers 1 and 2, 400 bp for primers 1 and 3) were obtained. Bands of the same size were also obtained with primers specific for MDC9 and MDC15. Subcloning and sequencing of the PCR products confirmed that that they indeed corresponded to murine MDC9 and MDC15, respectively. Sequencing of the PCR products obtained with degenerate primers also revealed the presence of MDC9 and MDC 15. No additional or novel MDCs were identified. This suggests that the predominant MDCs expressed in this tissue are MDC9 and MDC15. However, other MDCs may be present in smaller amounts, or some novel MDCs may be less conserved and may not hybridize with the primers as efficiently as the known MDCs.

Antibodies to synthetic peptides, recombinant protein fragments expressed in bacteria, and mammary tumor cell surface proteins:

As outlined in the original proposal, we have raised polyclonal antibodies to synthetic peptides and to recombinant MDC protein fragments expressed in bacteria. Even though these antibodies recognized the respective immunogens, they did not cross-react with native MDC proteins and did not produce specific ataining patterns of breast cancer tissues. This may be due to the complex folding of the MDC proteins, which is difficult to reproduce with short synthetic fragments or with protein fragments expressed in bacteria. We have also attempted to express MDC fragments in mammalian cells (human embryonic kidney 293 cells), but the expression levels were to low to allow purification of the recombinant proteins in the amounts needed for raising antibodies (100-200 micrograms).

Therefore, we have changed our strategy by attempting to raise monoclonal antibodies to all cell surface proteins, or to protein fractions enriched in metalloproteases, and to screen for antibodies that react with MDC proteins. To this end, we have now established an efficient novel method for raising monoclonal antibodies in rabbits. This method depends on the availability of a stable subclone of the rabbit plasmacytoma cell line 240E, originally described by K. Knight and colleagues (Spieker-Polet et al., 1995). We immunized rabbits with tumor cells obtained from a transgenic mouse mammary tumor model. We used primary cultures of tumor tissue that had been dissociated with collagenase, and injected several rabbits subcutaneously in the hind legs. After 4 injections at 1-week intervals, we collected blood and found that the serum contained a high concentration of antibodies reactive with the tumor cells. We then removed the spleens as well as the inguinal and popliteal lymphnodes and recovered the lymphocytes. The lymphocytes were suspended in 10% DMSO/90% fetal bovine serum and frozen in liquid nitrogen. We had previously shown that rabbit lymphocytes can be frozen and stored in liquid nitrogen without any loss of fusion capacity. Aliquots of the lymphocytes were fused with 240E cells, and plated in 96-well plates. After two weeks, hybridoma supernatants were screened by immunohistochemistry of frozen mouse mammary tumor tissue, to detect antibodies that produced specific tissue staining patterns. A variety of different staining patterns was observed, suggesting that many of the hybridomas produced distinct antibodies. We have used this system to produce several hundred hybridoma clones that stably secrete monoclonal antibodies reactive with mouse mammary tumors. An overview of the results is shown in the table below.

Total number of wells (30 plates)	2880
Number of wells with hybridoma colonies	1152
Number of wells with positive reaction in immunohistochemistry	157
Number of antibodies preferentially reacting with tumor cells	80
Number of antibodies reacting with both normal epithelium and tumor cells	57
Number of antibodies reactive with myoepithelial cells	10
Number of antibodies reactive with stroma	11
Number of antibodies reactive with cell-cell junctions	2
Number of antibodies detecting secreted molecules	5
Number of antibodies specific for necrotic areas of tumors	2

We have further characterized several of these antibodies and have found that they recognize the respective antigens with high affinity and specificity. Many of the antibodies can be used for western blotting, immunoprecipitation, immunohistochemistry, and immunocytochemistry. Thus, these antibodies are ideal reagents for characterizing novel proteins. In order to identify the antigens recognized by these antibodies, we have developed a rapid expression cloning protocol. We have

obtained a custom-made cDNA library (Edge Biosystems, Gaithersburg, Maryland) made from the mouse mammary tumor model described above. Pools of cDNA clones from this library were transfected into mammalian cells, and screened for reaction with the respective antigens. Six different clones were identified and sequenced. Comparison of these sequences with the genbank database revealed the identity of antigens recognized. In all cases, the size of the bands recognized by these antibodies in western blotting agreed with the expected properties of the proteins identified by expression cloning. The following table summarizes the proteins identified by expression cloning:

CD24; mucin-like PI-linked peripheral membrane protein
MFG-E8; RGD-containing adhesion protein present at the cell surface and in milk fat
mCaCl-1; calcium-dependent epithelial chloride channel/adhesion protein
BAP-31; ER-membrane protein involved in apoptosis
Cytokeratin 19; cytoskeletal protein
Fibronectin; extracellular matrix protein

So far, none of the antibodies identified a protein with properties expected of MDCs. Since MDCs probably constitute a minor fraction of total cell surface proteins, it may be necessary to immunize rabbits with protein fractions highly enriched in MDCs. To this end, we are using metal-chelate chromatography to enrich for cell-surface proteins interacting with Zn. Since most MDCs contain zinc-binding metalloprotease domains, and most other metalloproteases are secreted proteins, this approach is expected to highly enrich for MDCs.

We have also initiated a novel approach to raising anti-MDC antibodies. This approach takes advantage of recent advances in DNA immunization. It is now well established that intradermal injection of cDNA expression constructs results in expression of the protein and elicits a strong humoral immune response to the expressed protein. Thus, we will immunize rabbits with MDC9 and MDC15 expression constructs, and test the immune serum for the presence of antibodies reacting with the respective proteins. Polyclonal antibodies obtained in this way may prove to be specific reagents allowing the detection of the protein in tissue sections. In addition, we will obtain hybridomas from the spleens of immunized rabbits, and screen for antibodies that breast cancer tissue sections. These antibodies will then be further characterized by western blotting and immunoprecipitation.

CONCLUSIONS:

We have identified MDC9 and MDC 15 as the major MDC proteins expressed in mammary tumors of MMTV-mT transgenic mice. Further characterization of the role of these proteins in breast cancer is dependent on raising antibodies that recognize the respective native proteins in tissue sections. We have raised antibodies to synthetic peptides and recombinant MDCs, but these antibodies did not react with the native proteins. We have also raised antibodies to mammary tumor cell surface antigens, and have identified several breast cancer antigens. We are continuing to screen these antibodies for reaction with MDCs.

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APPENDICES: None

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CAR APPROVAL LETTER

Project # 96013425

June 3, 1999

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Dept.: Medicine
Phone No.: 206-4889/206-4123

Study Title: Characterization of Mouse Integrins

APPROVAL NUMBER: A6169-13425-03

Approval Date: 05/27/99
Expiration Date: 05/30/00

This number is a UCSF Committee on Animal Research (CAR) number which should be used for ordering animals for this study. This number may only be used by the principal investigator and those listed as participants included in the protocol and should be referenced in any correspondence regarding this study. The committee must be notified in writing of any changes to the approved protocol including changes in personnel.

Please distribute the final approved protocol to all individual participants so that they are familiar with the procedures that have been approved. Please remember that all personnel are to be fully trained before undertaking any procedures independently.

If you have any questions, please contact the Committee on Animal Research office at (415) 476-2197, Suite 315, Laurel Heights, Box 0962, or by electronic mail at carora@itsa.ucsf.edu.

SPECIES NAME	TOTAL NUMBER APPROVED					
	Purchased			Bred		
	Category A	Category B	Category C	Category A	Category B	Category C
Mice	0	0	0	0	30	0
Rabbits	0	30	0	0	0	0

John M. Taylor

John M. Taylor, Ph.D., Chairman
Committee on Animal Research